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The Membrane (M_1) Protein of Influenza Virus Occurs in Two Forms and Is a Phosphoprotein

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The membrane (M_1) protein of influenza virus was found to be heterogenous and to occur in two forms in the virus particle. The two forms of M_1 were found in virus which was produced both early and late after infection and in infected cells. The two forms could be separated on polyacrylamide gels under specific conditions. The two components of M_1 contained similar tryptic peptides. However, a small proteolytic difference between the two proteins could not be ruled out. Both M_1 proteins were present in phosphorylated form in the virus particle. The phosphorylated M_1 components were not readily distinguished from phosphorylated nonstructural protein (NS_1) when cytoplasm of infected cells was analyzed on polyacrylamide gels. The two phosphorylated M_1 components could, however, be detected in infected cells by immunoprecipitation. One M_1 component contained only phosphoserine whereas the second contained phosphoserine and a small amount of phosphothreonine as well. In addition to the phosphorylated nucleoprotein and M_1, a third phosphorylated protein was routinely detected in virus particles. It was a surface component of the virus, since it could be removed from whole virus with chymotrypsin and contained phosphate at serine residues. The identity of this component was not known.

The lipid-containing viruses which bud from eucaryotic cells have been important tools in the study of membrane biogenesis. Influenza virus is a model system for studying membrane formation, and we chose to study the membrane (M_1) protein, which is the major component of the virus. The paramyxoviruses and rhabdoviruses contain equivalent types of M proteins, which must play a pivotal role in the assembly of the virus particle. The M protein must recognize the site on the plasma membrane where insertion of the external glycoproteins has occurred and must also entrap the ribonucleoprotein to give an infectious unit. Therefore, the characterization of these proteins is important in studies dealing with the mechanisms involved in the formation of membranes and the budding process of these viruses.

The influenza virus M_1 protein is situated below the lipid bilayer of the virus and appears as a tightly adherent sac that surrounds the ribonucleoprotein (3, 35). In vitro evidence suggests that M is part of the lipid bilayer of the virus (4, 11, 12, 17). Isolated M_1 has a high affinity for lipid and can be inserted into lipid vesicles by the detergent dialysis method (4, 11) or merely by shaking aggregates of M_1 with preformed lipid vesicles (11). Two regions of M_1 embed into bilayers in vitro (12). More direct evidence has been obtained that M is part of the lipid bilayer in intact virus by the use of activatable hydrophobic probes. These type of probes partition into lipid bilayers and cross-link to all components embedded in the lipid when exposed to UV light. Such probes bind to the hemagglutinin (HA), the neuraminidase, and M_1, indicating that M_1 is an integral part of the viral membrane (12).

The M_1 protein of influenza virus contains many areas in its sequence which are made up of uninterrupted stretches of neutral and hydrophobic amino acids (1, 19, 39). This unusual sequence explains the high affinity of the M_1 protein for lipid, the solubility of M_1 in organic solvents (9), and probably the anomalous electrophoretic behavior of M_1 on polyacrylamide gels (13). M_1 migrates slower than the nonstructural (NS_1) protein induced in the infected cell, at concentrations of cross-linker to acrylamide below 1.2%. This is consistent with the molecular weights of these proteins. At concentrations of cross-linker to acrylamide above 1.2%, M_1 overtakes NS_1 and migrates faster. This behavior may be due to a disproportionate binding of sodium dodecyl sulfate (SDS) by hydrophobic stretches in M_1 (21, 24, 31). The localized binding of SDS may then affect the mobility of the protein when the pore size of the polyacrylamide is altered.

The primary structure of M_1 confers unique properties to the M_1 protein. In the data presented here, we show further that M_1 occurs in two forms in the virus and in the cell. These two forms are modified and are present in the virus and in the cell as phosphoproteins. The M_1 proteins are phosphorylated predominantly at serine residues.

MATERIALS AND METHODS

Virus and cells. The WSN strain of influenza virus was used. The virus was grown on chicken embryo fibroblasts (CEF). Virus was grown in the presence of a mixture of radioactive amino acids or methionine and purified from the fluids as previously described (9). Virus-specific proteins present in the infected cell were detected by infecting monolayers with 2 PFU per cell. Cells were labeled with ^{14}C-amino acids or [^{35}S]methionine from 5 to 7 h after infection. Monolayers were harvested at 7 h after infection.

Fractionation of CEF. Monolayers of CEF were washed with reticulocyte swelling buffer (RSB) consisting of 0.01 M Tris (pH 7.4), 0.01 M NaCl, and 1.5 mM $MgCl_2$ and harvested in 0.5 ml of RSB per monolayer. Cells were homogenized with 30 strokes of a Dounce homogenizer. Nuclei were pelleted at 1,000 rpm for 10 min. The cytoplasm was removed, and the crude nuclei were suspended in 2 ml of RSB. The nuclei were made 0.75% with respect to Nonidet P-40 and sodium deoxycholate. Samples were left on ice for 5 min and sedimented at 1,000 rpm for 10 min. Nuclear pellets were washed once with 2 ml of RSB and collected by sedimentation.

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Labeling virus and infected cells with radioactive phosphate. Confluent CEF monolayers were starved of phosphate for 17 h by incubating the cells in phosphate-free Earle medium containing 0.5% gelatin and 0.5% lactalbumin. Cells were washed with phosphate-free Earle medium and infected with a multiplicity of 2 PFU per cell of virus which had been dialyzed to remove the phosphate. After 30 min of adsorption at room temperature, cells were overlaid with phosphate-free medium. Radioactive inorganic phosphate (300 to 500 μCi per plate) was added immediately after infection or at 5 h after infection.

^{32}P-labeled virus was prepared by infecting monolayers as described above. The virus was purified from the fluids at 30 h after infection. Cellular debris was removed by centrifugation of the medium at 2,500 rpm for 10 min. The fluid was then overlaid onto a cushion of 30% sucrose in 0.1 M NaCl–0.01 M Tris (pH 7.6)–0.001 M EDTA, and the virus was pelleted by centrifugation at 25,000 rpm in an SW27 rotor. The virus pellet was resuspended and sedimented through a 30 to 60% gradient of sucrose. The virus band was collected after 17 h at 18,000 rpm in an SW27 rotor. The virus was diluted and pelleted at 40,000 rpm in a Ti 50 rotor.

Extraction of proteins into organic solvent. CEF monolayers were harvested into RSB and homogenized with a Dounce homogenizer, and nuclear and cytoplasmic fractions were prepared as described above. Nuclei and cytoplasm were made 0.5 M with respect to NaCl and shaken with two volumes of chloroform-methanol (CM; 2:1 [vol/vol]). Samples were centrifuged, and the interface containing the denatured protein was extracted with acidic CM as described previously (9). The M protein was extracted from purified virus into organic solvent in a similar manner.

Antisera. The M protein was isolated from virus and used for immunization of rabbits after being incorporated into liposomes. The isolation and incorporation into liposomes by the detergent dialysis method was carried out as described (11). The first immunization was done subcutaneously with Freund complete adjuvant. The subsequent three immunizations were in incomplete adjuvant. The animals were given a booster 1 month after the final immunization and bled 10 days later. Antisera to NS_1 crystals were a gift of M. Morrongiello (23).

Immunoprecipitation. Samples of cell extract or virus were solubilized with 2% SDS or 2% Sarkosyl and boiled for 2 min. One hundred microliters of sample in 0.2 M NaCl–0.01 M Tris (pH 7.6)–0.001 M EDTA–2% detergent was diluted with five volumes of buffer containing 0.1 M NaCl, 0.05 M Tris (pH 7.6), and 0.2% SDS or Sarkosyl. Theophilline was added to inhibit phosphatase (0.1 mg/ml), and phenylmethylsulfonyl fluoride was added to inhibit proteases (0.05 M final concentration).

Polyacrylamide gel electrophoresis. Polyacrylamide gels were formed with 15% acrylamide, containing 1% *N,N*-methylenebisacrylamide cross-linker (100 g of acrylamide; 1 g of *N,N*-methylenebisacrylamide). The running gel contained 0.37 M Tris-hydrochloride (pH 8.8) and 0.1% SDS, whereas the spacer consisted of 5% acrylamide in 0.125 M Tris-hydrochloride (pH 6.7) and 0.1% SDS. Electrode buffer was 0.03 M Tris–0.19 M glycine (pH 8.3)–0.1% SDS. Electrophoresis was at 33 mA and continued for 1 h after the bromophenol blue dye ran off the gel. Running gels were polymerized rapidly by incubating the solution of acrylamide at 37°C and then adding the ammonium persulfate and the *N,N,N',N'*-tetramethylethylenediamine.

Analysis of tryptic digests. Proteins were separated on polyacrylamide gels, and the bands were identified by autoradiography. The specific protein bands were cut out, and the gel was hydrated and fixed in methanol-water (5:5, vol/vol) to remove the SDS. The protein was eluted out into 44% formic acid. Samples were dried, suspended in 0.3 ml of formic acid, and dialyzed against 0.05 M ammonium bicarbonate (pH 8.8). Samples were digested with 20 μg of tolylsulfonyl phenylalanyl chloromethyl ketone trypsin (Calbiochem-Behring, La Jolla, Calif.) for 1 h at 37°C and for 2 additional h at room temperature. Samples were dried, resuspended, and spotted onto silica GHL plates (Analtech, Newark, Del.). Electrophoresis and chromatography were carried out as previously described (10). Tryptic peptides were detected by autoradiography.

Identification of phosphorylated amino acids. The phosphorylated amino acids were identified on samples that had been isolated from polyacrylamide gels. The virus M_1 proteins were isolated as follows. ^{32}P-labeled WSN virus was treated with CM (2:1, vol/vol) to remove the lipid. The protein was collected by sedimentation and shaken once more with CM and once with ether. The final protein pellet was suspended in 0.3 M NaCl–0.01 M Tris (pH 7.6)–0.001 M EDTA–1% SDS and boiled for 2 min. The RNA was hydrolyzed overnight at room temperature by adding 20 μg of RNase. Mercaptoethanol was added, and the sample was heated for 2 min at 100°C. The viral proteins were separated on 15% polyacrylamide gels.

Protein bands from virus or cell fractions were localized by autoradiography and cut out. These were fixed in methanol-water (5:5, vol/vol), dried, and rehydrated in 0.05 M ammonium bicarbonate (pH 8.8). Gels were homogenized, and trypsin was added (50 μg). Samples were digested for 1 h at 37°C and overnight at room temperature. The polyacrylamide was removed by sedimentation, and the supernatant containing the tryptic fragments was dried. The fragments were then hydrolyzed in 0.3 ml of 6 N HCl at 100°C for 1.5 h. The material was dried. Ten micrograms of standard phosphoserine in water and 10 μg of phosphothreonine were added to the sample, which was then spotted onto an Avicel cellulose plate (20 by 20 cm; Analtech). Samples were separated in the first dimension in acetic acid-pyridine-water (50:5:945, vol/vol) (pH 3.5) at 400 V with cooling, in a Shandon electrophoretic setup (Shandon Southern Instruments, Inc., Sewickley, Pa.). The amino acids were separated in the second dimension by electrophoresis in formic acid-acetic acid-water (25:87:888, vol/vol) (pH 1.9) at 400 V ^{32}P-spots were detected by autoradiography, whereas the phosphoserine and phosphothreonine standards were visualized by spraying the same plate with 0.2% ninhydrin in acetone and heating the plate for 3 min at 100°C.

RESULTS

Presence of two protein bands in the location of the M_1 protein in virus. The M protein, which is 27,500 daltons (1, 39), has recently been designated M_1 to distinguish it from a second protein of about 11,000 daltons, designated M_2 (19). These two proteins are transcribed from the same viral RNA by a shift in the reading frame (1, 15, 19). Whereas the M_1 protein is the major component of the virus particle, M_2 is a nonstructural protein present only in infected cells (19). Occasionally we have noted that the M_1 protein splits into two bands when the viral proteins are analyzed on gels, and we have investigated this phenomenon further. The nucleoprotein (NP) has already been shown to occur in two forms both in the cell and in the virus (2, 41).

WSN virus labeled with [^{35}S]methionine was purified, and the proteins were separated on 15% polyacrylamide gels.

Two bands were detected in the NP area, which is consistent with previous findings (Fig. 1, lane 1). Two strong bands were also visible in the M_1 area. In addition to M_1, there are two other proteins which migrate to the same area; one is the HA_2 polypeptide and the second is the NS_1 protein, which is synthesized in great abundance in the infected cell (16, 20, 30). NS_1 has not been detected in purified virus particles, although virus inocula may contain some NS_1 which may be present as a contaminant from disrupted cells (36). It was necessary, therefore, to identify the second strong band in the vicinity of the M_1 protein in our virus preparations.

Therefore, virus was treated overnight with chymotrypsin to remove the HA_2 and any surface contaminant. The virus was then sedimented through a cushion of 30% sucrose, and the pellet was solubilized and analyzed on gels. The HA_0, neuraminidase, HA_1, and HA_2 were clearly removed by the chymotrypsin, whereas the two bands in the M_1 area were unaffected (Fig. 1, lane 2). These two bands were thus internal components of the virus. Both proteins were extractable into acidic CM, which we use routinely for isolating the M_1 protein from the virus (Fig. 1, lane 3). The proteins were designated M_{1a} and M_{1b} for purposes of discussion in this paper.

The molecular weight of the M_{1a} protein was assumed to be 27,500, which is the molecular weight calculated for the M_1 protein from its nucleic acid sequence (1, 39). The apparent molecular weight of M_{1b} was about 24,500. However, this difference in migration between the two components was not necessarily due to an actual difference in the molecular weight of the proteins. M_{1a} did not always separate from M_{1b}, but separation of the two was more certain to occur if the acrylamide was rapidly polymerized by having been warmed to 37°C. It was also important to use a small volume of sample, such as 5 μl.

We then investigated whether M_{1a} and M_{1b} were found in virus harvested early after infection. CEF were infected with a multiplicity of 2 PFU per cell and incubated at 37°C with [^{35}S]methionine. The fluids were harvested at 8 h after infection, and fresh medium was added to the cells. The second medium was harvested at 24 h after infection. Cells were also infected, and fluids were harvested after 40 h of infection. The virus was purified and treated with chymotrypsin to remove the HA_2 component, which migrates between M_{1a} and M_{1b} and makes the analysis more difficult. The chymotrypsin-treated virus was pelleted through a cushion of sucrose, solubilized, and analyzed on gels. The early virus harvested after 8 h of infection contained both M_{1a} and M_{1b}. The 24- and 40-h viruses showed a similar pattern. The ratio of the two proteins was the same regardless of the time that the virus was harvested.

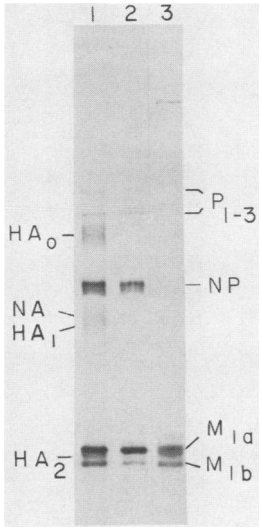

FIG. 1. Separation of [^{35}S]methionine-labeled WSN virus proteins on 15% polyacrylamide gels. Lane 1, WSN virus; lane 2, chymotrypsin-treated WSN virus; lane 3, CM extract of WSN virus.

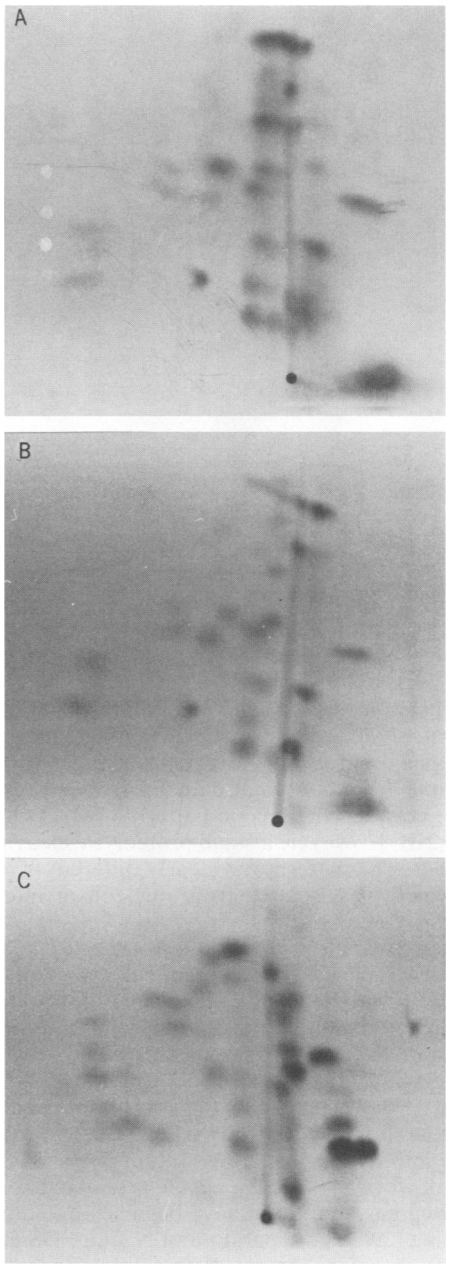

FIG. 2. Two-dimensional analysis of ^{14}C-amino acid-labeled tryptic digests of M_{1a}, M_{1b}, and NS_1 proteins on silica gel plates. (A) M_{1a}; (B) M_{1b}; (C) NS_1 from nucleoli of infected cells.

Tryptic digest peptides of the two forms of M_1. To determine the exact identity of the M_{1a} and M_{1b} proteins, WSN virus was labeled with a mixture of ^{14}C-amino acids, and the proteins were isolated from the virus into organic solvent

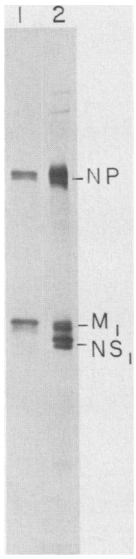

FIG. 3. Polyacrylamide gel analysis of [^{35}S]methionine-labeled WSN virus and WSN-infected cells. Lane 1, WSN virus; lane 2, infected cells labeled from 5 to 7 h after infection.

and separated on gels. Proteins were eluted from the gels and trypsinized, and the digest was analyzed on silica gel plates. This analysis was repeated a number of times with different virus samples. We could not detect a real and reproducible difference between the tryptic pattern of M_{1a} (Fig. 2A) and M_{1b} (Fig. 2B). However, a small proteolytic cleavage cannot be ruled out. The pattern was clearly different from the NS_1 protein which was isolated from nucleoli of infected cells (Fig. 2C).

Presence of two forms of M_1 in infected cells. The presence of two forms of M_1 in infected cells was investigated. Infected CEF were labeled with [^{35}S]methionine from 5 to 7 h after infection, solubilized, and analyzed on gels. A separation of M_1 into two bands was effected when whole cells were analyzed on gels, if the sample volume was kept small (Fig. 3, lane 2). Under the conditions which allowed for the separation of M_1 into two components, the NS_1 also separated into two species (Fig. 3, lane 2). The NS_1 protein consists of several species which are phosphorylated to different degrees, and these have been separated by isoelectric focusing (29). The separation of NS_1 in SDS gels seen in Fig. 3 may, therefore, be based on a difference in phosphorylation rather than on an actual difference in molecular weight.

Phosphorylated state of the M_1 protein in virus. The possibility that M_1 was a modified protein was investigated. Specifically, we determined whether the M_1 protein was phosphorylated. The virus was grown in the presence of $^{32}P_i$ and purified. The lipid was removed, the RNA was hydrolyzed with RNase, and the viral proteins were analyzed on gels. The NP and the M_1 proteins each separated into two bands when [^{35}S]methionine-labeled virus was run alongside of the ^{32}P-sample (Fig. 4, lane 1). Separation of the ^{32}P-labeled virus indicated that phosphate was associated with the NP as previously described (26–28). The phosphate was associated predominantly with the larger NP polypeptide (Fig. 4, lane 2), and this is consistent with the recent findings of Almond and Felsenreich (2). Lane 2 of Fig. 4 also shows that phosphate was associated with the two forms of the M_1 protein. The M_1 species seemed to be phosphorylated to about the same degree. Both were extractable into acidic CM.

In addition to the NP and M_1 proteins, another unidentified component was phosphorylated. This component migrated somewhat slower than the HA_0 component (Fig. 4, lane 2, component X) and could be removed if the ^{32}P-labeled virus was treated with chymotrypsin before it was analyzed on gels. It was, therefore, an external protein. It is not known if the protein is a specific viral component or a contaminant.

Detection of phosphorylated M_1 components in infected cells. The cytoplasm and nuclei from infected cells were analyzed for phosphorylated M_1 proteins. Cells were starved of phosphate overnight, infected, and labeled with [^{32}P]phosphate for 7 h. Cytoplasm and nuclear fractions were analyzed directly on gels without removing the nucleic acids or phospholipid. Many ^{32}P-labeled proteins could be detected in the uninfected control cells. These proteins were also detected in the infected cells. However, a very prominent band appeared in the infected cell which was not present in the control, and this band migrated to the position where the M_1 and NS_1 proteins were found. We were unable to distinguish phosphorylated M_1 from NS_1, which had previously been shown to be a phosphoprotein (28, 29). Nor was the NP detected in phosphorylated form under these conditions.

Antisera, which were made in rabbits against M_1 protein bound to lipid vesicles, were used to detect phosphorylated M_1 in the infected cell. Antisera specific for NS_1 were also used to detect phosphorylated NS_1. Whereas preimmune sera did not immunoprecipitate virus-specific proteins, antisera to NS_1 precipitated phosphorylated NS_1 which was present in the infected cell (Fig. 5, lane 2). The antisera to M_1 precipitated M_1 from [^{35}S]methionine-labeled cells and were specific for this component (Fig. 5, lane 5). Although more M_{1a} protein seemed to be immunoprecipitated in this preparation than is usually seen, both M_{1a} and M_{1b} are generally precipitated by the antiserum. When antiserum to M_1 was used on cytoplasm from infected and ^{32}P-labeled cells, the two bands of M_1 were immunoprecipitated (Fig. 5, lane 6).

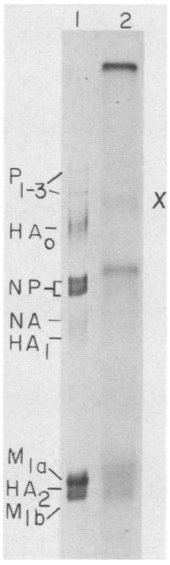

FIG. 4. Polyacrylamide gel analysis of [^{35}S]methionine- and [^{32}P]phosphate-labeled WSN virus. Lane 1, [^{35}S]methionine-labeled virus; lane 2, [^{32}P]phosphate-labeled virus.

Therefore, although the M_1 components could not be detected in phosphorylated form when whole cytoplasm was analyzed on gels, the proteins were present in phosphorylated form in the infected cell and could be detected by specific antisera.

Identification of phosphorylated amino acids in M_1 proteins. The phosphorylated amino acid species of the two M_1 components were identified and were compared with the phosphorylated residues of the NP and NS_1 components. The specific proteins were isolated from gels, hydrolyzed, and analyzed for phosphorylated amino acids on cellulose thin-layer plates. The M_{1a} component contained phosphoserine (Fig. 6A). The M_{1b} protein contained phosphoserine also, but a trace amount of phosphothreonine was detected (Fig. 6B). The NS_1 protein isolated from nucleoli contained only phosphothreonine (Fig. 6C), in agreement with the finding of Privalsky and Penhoet (29). A pool of NP polypeptides contained phosphoserine as previously reported (29), but a small amount of phosphothreonine could also be detected (Fig. 6D). It is not clear why the M_1 proteins separated into two components on gels, but it is possible that the separation is based in part on a difference in phosphorylation or is due to a slight proteolytic cleavage of one of the components.

DISCUSSION

The M proteins of the myxo, paramyxo, and rhabdoviruses play a central role in the assembly of these viruses. These proteins and their interactions with other viral components have only been partially characterized, although they are major proteins in all three groups of viruses (7, 33, 34, 38). The M proteins of vesicular stomatitis virus and influenza virus both interact with the viral lipid bilayer. The M protein of vesicular stomatitis virus interacts with lipid by hydrophobic and electrostatic bonds and does not penetrate into the bilayer (25, 40). The lack of partition into lipid bilayers is consistent with the sequence of that protein,

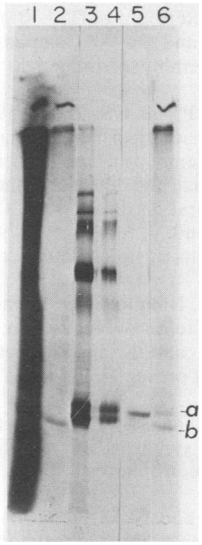

FIG. 5. Polyacrylamide gel analysis of immunoprecipitates from infected cells. Lane 1, [^{32}P]phosphate-labeled, infected CEF; lane 2, ^{32}P-immunoprecipitate from CEF formed by antiserum to NS_1; lane 3, [^{35}S]methionine-labeled cytoplasm from infected CEF; lane 4, [^{35}S]methionine-labeled WSN virus; lane 5, [^{35}S]methionine-labeled immunoprecipitate formed with antiserum to M_1; lane 6, ^{32}P-labeled immunoprecipitate from cells formed with antiserum to M_1.

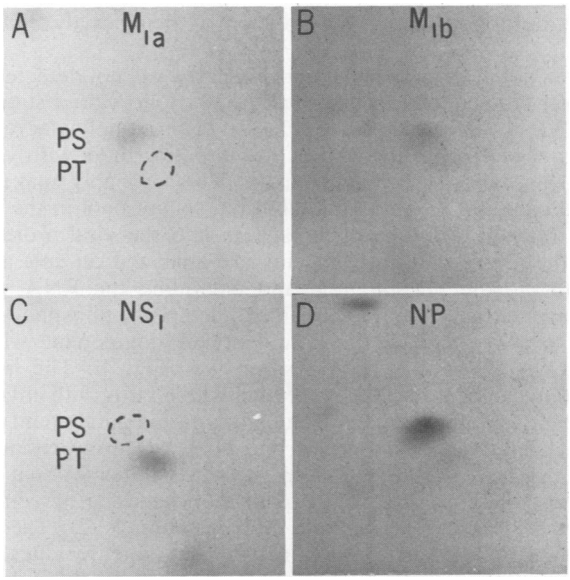

FIG. 6. Two-dimensional analysis on cellulose thin-layer plates of ^{32}P-labeled amino acids present in the M_1 components, the NP and NS_1 proteins. Phosphoserine and phosphothreonine markers were mixed with the samples before separation and identified by staining with ninhydrin. The markers are outlined in the panels at the left only.

which shows that there are no unusually long stretches of neutral and hydrophobic amino acids in the protein (32). The M_1 protein of influenza virus, on the other hand, is embedded into the lipid bilayer (12). This protein contains a number of stretches of neutral and hydrophobic amino acids in its sequence (1, 19, 39). The high affinity of influenza virus M_1 for lipid and the capacity of aggregated M_1 to readily partition into lipid bilayers (11) suggest that the protein inserts into membranes immediately after synthesis. The M_1 protein, already bound to membranes, then seeks the site where the HA and neuraminidase have been inserted at the plasma membrane site. This model would be consistent with the data showing that M_1 could not be chased from polysomes into smooth membranes and was always membrane bound (14, 22).

We investigated still another characteristic of influenza virus M_1 which may be pertinent to the mechanism of assembly of this virus. We had noted that the M_1 protein often separated into two bands when virus proteins were separated on gels. Since it was not clear whether this was a second form of M_1 or a different protein altogether, we investigated and found that M_1 is found in two forms in the virus and in the infected cell. The two forms of M_1 can best be separated when the acrylamide is polymerized rapidly and when small volumes of sample are applied to gels. The two forms of M_1 in the virus contain the same tryptic peptides. We cannot, however, rule out a small proteolytic cleavage between the two. Further analysis indicates that the two forms are modified by phosphorylation. One form of M_1 contains phosphate at serine residues only, whereas the second contains phosphate at serine residues and, to a small degree, at threonine residues as well. This difference in phosphorylation may be partly responsible for the difference in migration on gels. We tried to localize the exact site on M which is phosphorylated. Our preliminary efforts indicate that the phosphorylated tryptic peptide remains at the origin when the tryptic fragments are analyzed under the condi-

tions described here. This fragment is being analyzed further.

It is not clear why phosphorylated M_1 was not detected in earlier studies which described the phosphorylated state of the NP in the virus particle (27, 37). We routinely starve the cells of phosphate for 17 h before infecting them with virus which is also free of phosphate. This probably makes a difference in the size of the cold phosphate pool in the cell and facilitates uptake of phosphate into the viral proteins. Furthermore, the viral proteins are analyzed on gels after removing the phospholipid and degrading the RNA with RNase. In this way, it is possible to detect the phosphorylated NP, M_1, and a third unidentified component which migrates above the HA_0 position (see Fig. 4). The latter protein can be removed by treating whole virus with chymotrypsin, and it is not clear if the protein is a contaminant or a viral component; it contains phosphate at serine residues. Our analysis of the NP from the virus indicates that the larger NP is predominantly phosphorylated, in agreement with the findings of Almond and Felsenreich (2). The NP contains phosphate predominantly at serine residues, as reported previously (29), but a certain amount of threonine is also phosphorylated (see Fig. 6D).

The function of phosphorylation of M_1 is unknown. M is an integral membrane protein, and phosphorylation of structural membrane components may be important in the biosynthesis and assembly of membranes. Phosphorylation of major membrane proteins could alter the fluidity of the lipid bilayer and lead to rearrangement of membrane components (8), thus allowing for assembly. The introduction of a negatively charged group at a specific location on the M_1 protein could have an effect on the assembly of this or other viral proteins into the lipid bilayer. Alternatively, the phosphorylation may influence the interaction of the protein with the ribonucleoprotein during assembly or during transcription in the cell. It is known that M_1 interacts with the ribonucleoprotein in vitro to inhibit transcription (42). The reason for phosphorylation of the M protein in the rhabdo (5, 6), paramyxo (18), and myxoviruses is not clear, but the presence of phosphate on this component in all three groups of viruses argues for some specific function.

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